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<b>(54) Title:</b> SELECTING AMOUNTS OF PLATELET RELEASATE FOR EFFICACIOUS TREATMENT OF TISSUE			
<b>(57) Abstract</b>  A method of making a platelet releasate product is disclosed involving performing an assay on a platelet releasate sample for a component of the releasate and forming platelet releasate product by comparing the assay results to a predetermined range of amounts of the component to be contained in the product. A method of treatment of tissue is disclosed involving the topical application of such product.			

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SELECTING AMOUNTS OF PLATELET RELEASATE  
FOR EFFICACIOUS TREATMENT OF TISSUE

FIELD OF THE INVENTION

5 This invention relates to selecting amounts  
of platelet releasate for efficacious treatment of  
tissue.

BACKGROUND OF THE INVENTION

Tissue repair or "wound healing" involves  
many cellular and biochemical reactions which transform  
10 quiescent connective tissue and epidermal cells into  
rapidly dividing, rapidly moving cells. This  
transformation involves chemotaxis (movement of cells),  
mitogenesis (division of cells), and increased protein  
synthesis. Epidermal cells, fibroblasts, and capillary  
15 endothelial cells are involved in the formation of  
new tissue. Epidermal cells migrate and divide to  
form new skin (epithelium) at the site of tissue repair;  
fibroblasts move and divide to produce the matrix  
which fills the repair site (granulation site); capillary  
20 endothelial cells migrate and divide to produce new  
capillaries which revascularize the fibroblast/collagen  
matrix.

The process of cellular migration and mitosis  
is under the control of several biochemical agents.  
25 These locally-acting agents act on the various cells  
to direct their movement and division.

Each of these biochemical agents is

- (1) chemotactic (i.e., a chemoattractant) and causes migration (chemotaxis) of a certain type of cell;  
(2) mitogenic (i.e., a mitogen) and causes division (mitogenesis) of a certain type of cell; and/or  
5 (3) angiogenic (i.e., an angiogenic factor) and causes formation of new capillaries (migration and division of capillary endothelial cells). The chemotactic, mitogenic or angiogenic character of a biochemical agent is generally determined by testing the agent  
10 in various known assays for chemotaxis, mitogenesis and angiogenesis. Some of these assays are described below. Additional assays are expected to be developed in the future which will be directed at the same characteristics but perhaps will more clearly define  
15 or measure the presence of the characteristic.

Platelet-Derived Growth Factor (PDGF) is a well-characterized, 30,000 dalton dimeric glycoprotein with mitogenic and chemoattractant activity for fibroblasts, smooth muscle cells and glial cells.  
20 In the presence of PDGF, fibroblasts move into the area of tissue needing repair and are stimulated to divide in the wound space itself. The cells exposed to lower PDGF concentrations (approximately 0.5-1 ng/ml) are stimulated to move and, as they move to environments  
25 having higher concentrations of PDGF (approximately 1-2 ng/ml), they are stimulated to divide.

The process of neovascularization or angiogenesis (new capillary formation) is stimulated by angiogenesis factors. The angiogenesis factor  
30 recoverable from platelets (PDAF) is a pure chemoattractant without mitogenic activity for capillary endothelial cells, which stimulates them to move through a gradient towards the source of the angiogenesis factor. Once the capillary cells start to move from  
35 the parent capillaries, they start to divide. This division is probably under the control of autocrine growth factors produced by endothelial cells, which

have been found to be of the basic fibroblast growth factor (FGF) variety.

The combination of fibroblast migration and mitosis and endothelial cell migration and mitosis produces granulation tissue. Granulation tissue also is rich in neutrophils and monocytes which have been brought to the site of repair by the presence of C5A from complement activation and transforming growth factor beta (TGF-B) from platelets. The presence of these phagocytic cells decreases the contamination and prevents overt infection.

TGF-B is a 25,000 dalton (112 amino acid) polypeptide that has a function in the synthesis of fibrin-collagen. TGF-B inhibits the division of fibroblasts and increases their matrix production. Whether TGF-B stimulates or inhibits division is a function of the entire set of growth factors operating in the tissue. In the presence of PDGF, TGF-B usually stimulates division while it usually inhibits division in the presence of epidermal growth factors (described below).

After granulation tissue is formed, epidermal cells migrate from the cut skin edge over the granulation tissue to form a new skin layer, which then matures into normal skin. This cellular activity is at least partially under the control of platelet-derived epidermal growth factor (PDEGF) which is a chemoattractant for epidermal cells.

In summary, the process of tissue repair or "wound healing" is under the control of at least four growth factors: TGF-B, PDGF, PDAF and PDEGF. The presence of these growth factors in the tissue to be repaired produce fibroblast migration and mitosis, endothelial cell migration and subsequent mitosis, and epidermal migration and mitosis. The end result is the filling of wound space with a granulation tissue followed by reepithelialization and skin maturation.

Two principal sources of these factors for natural healing of tissue are platelets and macrophages. When tissue is damaged in the body, platelets are released by the presence of thrombin generated by the activation of the coagulation process. These platelets then release PDGF, PDAF, PDEGF, TGF-B and platelet factor 4 (PF-4 is a chemoattractant for neutrophils and monocytes) and stimulate release of complement C5A. The PDGF, PDAF, PDEGF and PF-4 themselves contribute directly to healing the wound as described above, while TGF-B and C5A attract macrophages to the damaged site. Macrophages also release same or similar mitogens, chemoattractants, and angiogenesis factors once they have been summoned to the area of tissue repair. However, to date, macrophages are not known to produce EGF-like activity.

Common reasons for the failure of nonhealing wounds to improve are: infection, poor cellularity, few fibroblasts, no new capillaries and few inflammatory cells. In contrast, healing wounds are characterized by mononuclear and macrophage cell infiltrates, dividing fibroblasts and numerous capillaries.

Knighton, et al, Ann. Sur. 1986, 204:322-330, incorporated in its entirety herein by reference thereto, treated 49 patients with chronic nonhealing cutaneous ulcers using autologous Platelet Derived Wound Healing Formula (PDWHF) in a microcrystalline collagen salve. The wounds had been treated an average of 198 weeks with conventional treatment. Mean healing time to 100% epithelization was 10.6 weeks direct correlation to 100% healing was related to initial wound size and initiation of PDWHF therapy. There was no abnormal tissue formation, keloid or hypertrophic scarring reported.

In a double-blind study, Knighton et al, Tissue Repair Symposium at Tarpon Springs, Florida May 1987, which is incorporated herein in its entirety



by reference thereto, compared wound healing using PDWHF in a collagen base to a placebo. All 24 patients received wound care according to a standard protocol. The 13 patients in the PDWHF group had healing to 100% epithelization in 17 or 21 wounds after 8 weeks of therapy; of 11 patients in the placebo group only 2 of 13 had wounds that reached 100% epithelization. The placebo failures were then treated with PDWHF and their wounds healed in an average of 7.1 weeks. The unhealed PDWHF treated patients continued on PDWHF and achieved 100% epithelization in an average of 5.8 additional weeks of treatment.

The pretreatment records for a group of patients seen at the University of Minnesota Wound Care Clinic were provided to a panel of three nationally recognized experts in amputation, diabetic foot care and vascular surgery. The 136 wounds in 73 patients were graded on a severity scale of 1 through 6 (partial thickness to full thickness with gangrene). PDWHF therapy had been initiated in each case. Over 75% had wound grades of 3 or higher. Seventy (70%) of the limbs evaluated were considered to be at significant or definite risk of amputation. Of the 26 limbs scored at no risk of amputation, none required amputation. Over 90% of the 53 limbs considered to be at significant risk of amputation were salvaged. Limbs scored at requiring immediate amputation (n=9) had a salvage rate of 86%. Applying PDWHF to wounds on a daily basis promoted granulation tissue formation and epidermal cell growth stimulation. Chronic nonhealing wounds have been healed to fully functional skin.

In the foregoing studies of wound treatment, the PDWHF formulation was based on releasing  $10^9$  platelets into medium to a final volume of 1 ml. No attempt was made to adjust the amount of platelet releasate to compensate for variation from donor to donor, or from time to time for a particular donor, of the potencies

of the wound healing factors contained in such platelet  
releasate. It is an object of the present invention  
to select amounts of platelet releasate for efficacious  
treatment of tissue, giving due regard to variations  
5 of the potencies of healing factors contained in the  
releasate.

#### BRIEF SUMMARY OF THE INVENTION

In accordance with the invention, tissue  
may be treated by applying topically a platelet releasate  
10 product containing a selected amount of platelet  
releasate. The platelet releasate product preferably  
is applied to tissue to be treated in an amount sufficient  
to cause, if desired, migration and/or division of  
fibroblasts, capillary endothelial cells and/or epidermal  
15 cells such that the division or migration of the cells  
contributes to the formation of granulation tissue,  
capillaries and/or epithelium in the area of treatment.

The present invention provides a method  
for making a platelet releasate product useful in  
20 treatment of tissue. An assay is performed on a platelet  
releasate sample, where the assay indicates the amount  
of a component present in the platelet releasate sample.  
The "component" preferably is a wound healing factor  
or its amount correlates to the amount of desired  
25 wound healing factors present in platelet releasate.  
The assay may be an immunoassay for detecting the  
amount or presence of such components or may be any  
other method for determining the presence or amount  
of the component such as the use of HPLC. Based on  
30 the results of the assay, a platelet releasate product  
is formed which contains a selected amount of platelet  
releasate. The amount is selected by comparing the  
amount of the component in the platelet releasate  
sample to a predetermined range of amounts of the  
35 identical component to be contained in a releasate  
product. The present invention further provides a  
method of treating tissue whereby the foregoing releasate

product is applied topically to tissue.

An alternative method of making a platelet releasate product, and topical application of such product to tissue, involves performing an assay on  
5 a platelet releasate sample, where the assay indicates the amount of an activity of the platelet releasate sample.

The platelet releasate contained in the platelet releasate product and in the platelet releasate  
10 sample is preferably obtained from the same draw of platelets. Alternatively, the platelet releasate contained in the platelet releasate product and in the platelet releasate sample may be obtained from different draws of platelets from the same animal  
15 or human. As a further alternative, the platelet releasate contained in the platelet releasate product and in the platelet releasate sample may be obtained from a pool of platelets taken from singular or multiple draws of platelets from a single animal or human or  
20 from a group of animals or human donors.

The range of the amount of the component to be contained in a platelet releasate product may be predetermined so that the selected amount of platelet releasate contained in said platelet product is sufficient  
25 to cause substantial efficacy of treatment of tissue in a chosen percentage of treatments. For example, such treatment efficacy may be chosen to obtain at least a grade 2 functional assessment score, hereinafter defined, in greater than 50% of wounds treated.  
30 Alternatively, a range of amounts of an activity of platelet releasate product may be similarly based on a desired efficacy of treatment of tissue.

The components, for which a range of amounts is predetermined for such efficacy, may be  
35 Beta-thromboglobulin ("B-TG"), PDGF, PDAF, PF-4, basic FGF, acidic FGF, TGF- $\alpha$ , TGF-B, PDEGF and fibronectin. The activity may be fibroblast mitogenic activity

("FMA"), endothelial cell chemotaxis activity ("ECCA"), rabbit corneal assay activity ("RCAA") or keratinocyte cell chemotaxis activity ("KCCA").

Finally, the platelet releasate may be combined with a pharmaceutically acceptable carrier or diluent for such platelet releasate to form a platelet releasate product. Further, the product may be substantially free of blood or plasma contaminants and free of platelet ghosts or other materials contained in the platelets but not released by platelets.

"Treatment" includes wound healing, cosmetic or any other process in which it is desirable to promote angiogenic, mitogenic or chemotactic activity in the region of the tissue to be treated. Possible treatment applications of tissue include, without limitation, any of those listed in Table 1. Such treatments are topical in the sense that they apply a composition to the surface of a region or body of tissue, but are not applied systemically.

Table 1

Potential Applications for  
Treatment of Tissue

- I. Treatment of chronic non-healing cutaneous wounds
  - A. Ischemic wounds
    1. Diabetic wounds
    2. Ischemic wounds from atherosclerosis
    3. Wounds from arteriolar vasculitis
  - B. Venous stasis wounds
    1. Post-phlebitic syndrome
    2. Post-traumatic venous stasis
  - C. Pressure sores
    1. Sacral decubitus
    2. Ischial decubitus
    3. Heel and malleolar decubitus
    4. Other areas of pressure
  - D. Wounds from persisting cutaneous trauma

## II. Topical treatment of acute wounds

## A. Split thickness wounds

1. Skin graft donor sites
2. Abrasions such as those occurring  
from a motorcycle accident

## B. Full thickness skin loss

1. Degloving injury
2. Traumatic skin loss
3. Traumatic skin necrosis

## 10 III. Burns

## A. Split thickness skin graft donor site repair

B. Acceleratin of granulation tissue formatin  
and early debridement and grafting of skin15 C. Acceleratin of re-epithelization of second  
degree burnsD. Improvement of cosmetic result in skin grafting  
by prevention of chronic contracture

## IV. Revascularization of intact skin

## A. Necrobiosis lipoidica diabetorum

## 20 B. Radiation induced skin ischemia

## C. Phemphigus vulgaris

## V. Costmetic applications

## A. Hair Growth

## B. Skin renewing preparation

## 25 C. Wrinkle treatment

## VI. Treatment of acute surgical wounds

- A. When combined with a slow-release biodegradable  
delivery system, compositions may enhance  
the rate of normal wound repair by shortening  
the lag phase. The delivery system may apply  
compositions topically to any surgical wound,  
either in the skin, fissures or internal organs.

## VII. Internal surgical applications

- A. Compositions may be used to accelerate repair  
of internal surgical or traumatic wounds,  
including without limitation liver laceratins,  
kidney lacerations, splenic lacerations and

anastomoses of, for example, the bowel, colon, or biliary tree

- 5       B. Topical administration to internal wounds  
      such as traumatic wounds of the liver and  
      spleen
- C. Compositions may be applied to intra-abdominal  
      abscesses to accelerate repair. For example,  
      when an intra-abdominal abscess is drained  
10       percutaneously and the drain is left in place,  
      compositions could be injected through the  
      drain so as to topically apply the composition  
      to the surfaces of the cavity to accelerate  
      repair of that potential space.
- VIII. Veterinary applications
- 15       A. Accelerate surgical repair
- B. Accelerate repair of chronic non-healing wounds  
      such as those of horses
- C. Accelerate repair of horse long bone fractures
- D. A system could be devised to deliver compositions  
20       to treat wounds in cattle to keep the contraction  
      process from occurring and closing off the  
      mammary duct
- IX. Ophthalmologic applications
- 25       A. Accelerate healing of corneal ulcers
- B. Accelerate healing of corneal transplants
- C. Accelerate healing of other types of ophthalmologic  
      surgery
- X. Orthopaedic applications
- 30       A. Accelerate normal fracture healing
- B. Stimulate repair of non-unions
- C. Facilitate bone graft healing
- D. Stimulate repair after osteomyelitis debridement
- E. Compositions could be combined with prosthetic  
      material (such as joint replacement) to accelerate  
35       tissue in growth
- F. Accelerate repair of tendon and ligament injuries
- G. Stimulate incorporation of artificial tendons

## XI. ENT applications

- 5       A. Accelerate repair of mastoidectomy wounds  
          (which could be accomplished by topical  
          administration similar to that done presently  
          for chronic non-healing wounds)
- B. Combined with artificial prostheses (such  
          as tympanic membranes, tympanic membrane tubes,  
          or artificial eustachian tubes)

## XII. Plastic surgical applications

- 10       A. Control tissue remodeling (fill tissue defects  
          with new tissue)
- B. Stimulate ingrowth into prosthesis (i.e. breast  
          implants)
- C. Stimulate accelerated repair in flaps
- 15       D. Since the scar which results from the topical  
          administration of compositions is much more  
          cosmetically satisfactory than an unstimulated  
          scar, the material could be used topically  
          in scar revision
- 20       E. Accelerate repair of tendon injuries such  
          as those occurring in the hand

## XIII. Dental

- A. Accelerate repair of dry sockets
- B. Accelerate normal socket repair
- 25       C. Accelerate ingrowth into dental implants
- D. Stimulate gum growth at the tooth bone line

## XIV. Gastrointestinal applications

- A. When combined with the drug such as Sucralfate,  
          compositions may accelerate the repair of  
30       stomach and duodenal ulcers
- B. Compositions may accelerate repair of ulcerative  
          colitis in the colon when given as an enema
- C. Compositions may accelerate repair of  
          granulomatous colitis when given orally in  
35       a slow-release material

## XV. Vascular surgery

- A. Compositions (and especially angiogenesis

factor) when combined with an arterial graft, may stimulate new vaso vasora to form which would re-endothelize the graft from the capillary ingrowth

5 XVI. Artificial endocrine organ

- A. The angiogenesis factor may be used to stimulate recapillary ingrowth into tubes in an artificial endocrine organ which could be implanted into the body. The capillaries would be stimulated to grow through the tubes and the cells or islets could be grown on the outside of the tubes to allow use of a totally xenographic endocrine system

XVII. Stricture formation

- 15 A. Compositins may be combined with stents which are now being used in the esophagus, biliary tree, urethra, and ureters to stimulate angiogenesis and healing of a stented tubular structure which then decreases the restricture formation rate. The compositions may be delivered on the stent in a slow-release form such that the compositions are topically applied to the tissue surfaces surrounding the stent

25 Uses for compositions of the present invention include, without limitation, any of those listed in Table 1 of 1A.

Table 1A

Additional Potential Applications for  
Compositions of the Present Invention

- 30 I. Revascularization of myocardial infarctions
- A. Compositions may be injected into the center of a myocardial infarction by cardiac catheterization or percutaneously guided by magnetic resonance imaging in a slow-release system and accelerate infarct repair
- 35 B. Compositions may be targeted with liposomes



coated with anti-denatured collagen antibodies and given intravenously for migration to the site of a wound or myocardial infarction.

## II. Revascularization of neural injuries

5 A. Compositions may be injected into a cerebral infarct or spinal cord injury in a slow-release system to accelerate repair

B. As in I above, targeted liposomes carrying compositions may be used intravenously with  
10 anti-denatured nerve antibodies to migrate to the site of a neurologic injury

A chemical species has "chemotactic activity", "mitogenic activity" or "angiogenic activity", as those terms are used throughout this specification  
15 and the appended claims, when such species exhibits a positive response in the corresponding assays for angiogenesis, mitogenesis and chemotaxis described herein or similar assays available in the art or as later developed.

20

### DESCRIPTION OF FIGURES

FIG. 1 shows a plot of functional assessment versus ng/ml of B-TG in platelet releasate extract in a Florida study.

25 FIG. 2 shows a plot of functional assessment versus ng/ml of B-TG in platelet releasate extract in a Kansas City study.

FIG. 3 shows a plot of functional assessment versus ng/ml of B-TG in platelet releasate extract in a Minnesota study.

30 FIG. 4 shows a plot of B-TG versus PF-4 in platelet releasate extract.

FIG. 5 shows a plot of B-TG versus PDGF in platelet releasate extract.

35 FIG. 6 shows a plot of B-TG versus FMA activity in platelet releasate extract.

DETAILED DESCRIPTION  
OF THE INVENTION

Efficacy of treatment of tissue has been defined for chronic nonhealing cutaneous wounds.

5 Functional assessment grades 1-4 measure wound healing maturity based on the following ratings:

- (1) less than 100% epithelized; has drainage; needs a dressing.
- 10 (2) 100% epithelized; has drainage; needs a dressing for drainage control.
- (3) 100% epithelized; maturing skin with small amount of drainage; requires protective dressing only.
- 15 (4) 100% epithelized; 100% mature functional skin; does not require a dressing.

The preferred procedure for treatment of chronic nonhealing cutaneous wounds involves applying the platelet releasate product once a day, at the same time each day. The product should remain on  
20 the wound for at least eight hours before it is rinsed off. During the other 12 hours, when the product is not on the wound, saline wet to dry dressing should be applied to the area.

While it is preferred to prepare platelet  
25 releasate product for wound treatment purposes directly from a patient's own blood, the advantages of the invention may be achieved by using blood or outdated platelets from other sources. Using a patient's own blood is disclosed since it avoids exposure of the  
30 patient to possible hepatitis, AIDS or other contaminants from banked blood. Using a patient's own blood also eliminates most allergic reactions to foreign blood. An alternative source of the product may, however, be obtained from pedigree blood (i.e. for persons  
35 screened for hepatitis, AIDS, etc.) or from outdated human platelets, and either from a single source or a pool of a plurality of sources. Blood from other

species may also be used for human application. Finally, platelet product may also be utilized in veterinary applications by utilizing platelets derived from the animal itself, from another animal within the same species, or from another species.

#### EXAMPLE 1

60 ml whole blood was aseptically obtained from a source in 6 ml of acid citrate dextrose anti-coagulant (hereinafter ACD), or 1 ml ACD per 10 ml of whole blood. The blood was mixed well with ACD by inverting and rolling the syringe. Anti-coagulated blood samples were kept on ice until used in further processing.

The anti-coagulated blood was transferred to two sterile, siliconized 50 ml conical-bottom centrifuge tubes, evenly splitting the sample between tubes. The tubes were then centrifuged at 135 x g for 20 minutes at about 4°C. Upon completion of the centrifugation cycle, the rotor was allowed to coast to a stop. No braking was applied. The uppermost layer of the centrifuged sample, platelet-rich plasma (hereinafter PRP), was carefully transferred with a sterile pipette to another sterile, siliconized centrifuge tube. Drawing only 4-5 ml at a time minimized losses due to red blood cell contamination of the PRP. A platelet count of the PRP was then conducted using methods well known in the art.

The PRP was centrifuged at 750 x g for 10 minutes at about 4°C. The supernatant was discarded, being careful not to dislodge the platelet pellet. Using a sterile pipette, the pellet was resuspended by aspirating and expelling buffer containing 0.05 M HEPES (N-2-hydroxyethyl piperazine-n-2-ethane sulfonic acid), 0.03 M dextrose, 0.004 M KCl, 0.1 M NaCl, pH adjusted to approximately 6.5 at 28°C (hereinafter platelet buffer) to an approximate concentration of  $10^9$  platelets per ml of suspension.

The resulting platelet suspension was then activated with purified thrombin. Preferably, about 1 unit of thrombin per ml of platelet suspension was added to the platelet suspension and mixed. The platelets and thrombin were allowed to incubate at room temperature for about 10 minutes. After incubation, the resulting platelet aggregate was broken up by aspirating and expelling the suspension with a sterile pipette.

Alternatively, the platelet suspension may be activated with other activators that cause the platelets to release their contents. Other activators include collagen, preferably 6-100 ug of monomer collagen per ml of buffer containing 10% platelets, ADP, preferably 2-10 u molar in said buffer, epinephrine, preferably 25-450 u molar in said buffer, and arachidonic acid, preferably 35-50 u molar in said buffer.

As a further alternative embodiment, PRP can be activated with thrombin or otherwise before centrifugation. The activated PRP can also be incorporated into liquid or paste preparations as described below.

In the preferred embodiment, the resulting supernatant was centrifuged at 950 x g for about 5 minutes at about 4°C, thereby removing the released platelet ghosts and any fibrin contained in the suspension. The pellet formed by such centrifugation was discarded after the supernatant was extracted.

After removal of the platelet ghosts and fibrin, the remaining supernatant constitutes platelet releasate in platelet buffer, herein designated platelet releasate extract. The extract is frozen in 4 ml aliquots for storage or immediately used for assay or to make liquid or paste product as described below.

#### EXAMPLE 2

Platelet releasate extract may be prepared from platelets obtained from a blood bank or other source. Pheresis platelet concentrate may be obtained

from a blood bank and immediately processed. One unit of platelets will yield approximately 200 mls of PRP.

The concentrate may be processed to produce the activated platelet suspension in the same manner as the anti-coagulated patient blood sample is processed above, except that the PRP is centrifuged four times at 750 x g for 10 minutes at about 4°C, resuspending the platelet pellet in platelet buffer after each centrifugation. After the fourth centrifugation, the platelet pellet is resuspended in platelet buffer to an approximate concentration of 10<sup>9</sup> platelets/ml.

The platelet suspension is activated as described above and centrifuged at 950 x g for 10 minutes at about 4°C. The supernatant is extracted and centrifuged at 10,000 x g for 15 minutes at about 4°C to remove residual platelets and any fibrin. The pellet is discarded after the supernatant is extracted. The supernatant which is the platelet releasate extract is frozen in 4 ml aliquots for storage or immediately used to make into liquid or paste preparations as described below.

As a further alternative to production from blood bank platelets, PRP produced from banked platelets can be directly activated before centrifugation.

#### EXAMPLE 3

Platelet releasate product is preferably administered to the patient in a liquid form. Platelet releasate extract, i.e. frozen platelet releasate in platelet buffer, may be thawed to room temperature. A measured volume of extract at room temperature may be added to a centrifuge tube so as to yield, after addition of platelet buffer to the centrifuge tube, a preferred dilution. 0.5 ml lidocaine may be added before adding platelet buffer. In this case, the buffer acts as the carrier. Also, the extract may in some cases be used without further dilution.

EXAMPLE 4

As an alternative embodiment, platelet releasate product may be applied in a paste form, comprising the extract in a carrier substance which is biologically compatible with and acts as a temporary "depot" for the active components of the supernatant. A macromolecular substance such as microcrystalline collagen, e.g. Avitene<sup>®</sup> brand microcrystalline collagen commercially available from Alcon Laboratories, Inc., Forth Worth, TX is a suitable carrier.

To prepare the paste platelet releasate product, the extract was thawed and diluted as above for the liquid form. Appropriate amounts of extract may be pipetted into a jar of Avitene microcrystalline collagen and mixed with a sterile pipette to achieve uniform consistency. Alternatively, extract may be mixed with the microcrystalline collagen.

The jars containing the resulting paste platelet releasate product should be capped and put into plastic bags with ice or frozen for transport or storage until applied to the patient.

EXAMPLE 5

In a wound treatment study in Florida, U.S.A. involving 102 nonhealing cutaneous wounds, autologous platelet releasate product was used to treat the wounds according to the above treatment procedures. The platelet releasate product was produced as follows: the platelet releasate extract, produced according to the procedures of Example 1, was further diluted 1:100 to form platelet releasate product. Prior to topical application of each of the platelet releasate products, an immunoassay for beta-thromboglobulin, available commercially from Diagnostica Stago, Asnieres-Sur-Seine, France as ASSERACHROM B-TG, was performed on the product corresponding platelet releasate extract to determine the amount of B-TG contained in the extract itself. The wounds were graded for

functional assessment after completion of treatment.  
The amount of B-TG contained in the platelet releasate sample, which in this case was the extract, correlated to the success of the treatment by platelet releasate product as measured by the functional assessment:

5

Variables: B-TG VS FA  
Sample Size: 102  
Spearman R: 0.2427  
T-value: 2.5023  
2-Tail P: .014

10

FIG. 1 shows a representative plot of the data.

#### EXAMPLE 6

The foregoing example was repeated in a Kansas City, U.S.A. wound treatment study involving 86 wounds treated with autologous platelet releasate product. The amount of B-TG contained in the platelet releasate sample correlated to the functional assessment:

15

Variables: B-TG VS FA  
Sample Size: 86  
Spearman R: 0.3 508  
T-Value: 3.4328  
2-Tail P: 0.0009

20

FIG. 2 shows a representative plot of the data obtained.

#### EXAMPLE 7

Example 5 was again repeated at a Minnesota, U.S.A. wound treatment study involving 32 wounds treated with autologous platelet releasate product. The amount of B-TG contained in the platelet releasate sample correlated to the functional assessment:

25

Variables: B-TG VS FA  
Sample Size: 32  
Spearman R: 0.3629  
T-Value: 2.1329  
2-Tail P: 0.0412

30

FIG. 3 shows a representative plot of the data obtained.

35

Based on the foregoing examples of wound treatment, the success of wound treatment correlated

to the amount of B-TG contained in the extract used to produce the product. Accordingly, platelet releasate product should preferably be manufactured to have a predetermined range of the amount of B-TG in the product. Since the product is composed of dilutions of platelet releasate extract, the amount of platelet releasate in form of extract or otherwise used to constitute the product should be adjusted to account for the amount of B-TG contained in the releasate extract. This manufacture procedure will enable the product to have a desired amount of B-TG in the product, even though the amount of B-TG in releasate varies from donor to donor and from time to time for a particular donor.

As illustrated by FIGS. 1-3, if an average FA grade of 2 or more is desired, the amount of B-TG should be at least about 25 ng per ml of product. Of course, B-TG amounts in excess of 66 ng per ml of product give optimal healing within the range of amounts of B-TG investigated by the studies.

Alternatively, other components of platelet releasate may be used to manufacture platelet releasate product containing minimal or optimal amount of platelet releasate. These components include, without limitation, PDGF, PDAF, PF-4, basic FGF, acidic FGF, TGF-2, TGF-B, PDEGF and fibronectin. Examples 8-9 show the correlation between B-TG and PF-4 and PDGF.

#### EXAMPLE 8

41 autologous platelet releasate samples were assayed for B-TG by the above B-TG immunoassay and for PF-4 by a PF-4 immunoassay available commercially from Diagnostica Stago as ASSERACHROM PF-4. The amount of B-TG in the platelet releasate sample correlated to the amount of PF-4 as follows:

Variables:	B-TG VS PF-4
Sample Size:	41
Spearman R:	0.9148



21

T-Value: 14.1449

2-Tail P: &lt; 0.0001

FIG. 4 shows a representative plot of the data.

EXAMPLE 9

5 41 autologous platelet releasate samples were also assayed for B-TG according to the above B-TG assay and for PDGF according to the following assay protocol:

PDGF EIA PROTOCOL10 DAY 1:

1. Block Reaction Plates (R) (Dynatech Imm-1, round bottom)
  - a) Add 150 ul PT-20 (PBS TWEEN-20 .05%) per well.
  - b) Incubate R-plates for 60 min at 37°C covered.
  - 15 c) Aspirate R-plates and dry. Go to step 3.
2. Coat Quantitation Plates (Q) (Dynatech Imm-2, flat bottom)
  - a) Add 150 ul/well PDGFcsis (40ng/ml in coating buffer).
  - 20 b) Incubate Q-plates overnight at 4°C covered in ziplock bag.
3. After Blocking
  - a) Make sample dilutions in PBA-T/20 (PBS+1%BSA+.05%T-20) using polypropylene tubes. Mix well.
  - 25
4. Adding samples to R-plate
  - a) Add 60 ul/well Goat Anti-PDGF (dil in PBA-T/20) at 2 ug/ml.
  - b) Add 60 ul/well of diluted sample or standard.
  - 30 c) Incubate R-plates overnight at 4°C covered in bag.

DAY 2:

1. Q-plate Aspiration
  - a) Aspirate Q-plate.
  - 35 b) Block Q-plate with 150 ul/well PT-20 (1-2 hrs, 37°C).
  - c) Aspirate, wash 3X, air dry.
2. R-plate Transfer

- a) Transfer contents of R-plates to Q-plates (100 ul).
- b) Incubate Q-plates at room temp. for 30 min.
- c) Aspirate and wash.

5     3. Color Reaction

- a) Add 100 ul/well Rat ANTI-Goat-Peroxidase (1 ug/ml).
- b) Incubate 1 hr. at room temp.
- c) Aspirate and wash.
- 10    d) Add 100 ul/well substrate (Tetramethylbenzidine).
- e) Read plates.

The amount of B-TG contained in the platelet releasate sample correlated to the amount of PDGF as follows:

	Variables:	B-TG VS PDGF
15	Sample Size:	41
	Spearman R:	0.8103
	T-Value:	8.6359
	2-Tail P:	< 0.001

FIG. 5 shows a representative plot of the data.

20           As a further alternative, activities of platelet releasate may be used as the basis for manufacturing platelet releasate product containing a minimal or optimal amount of platelet releasate. These activities include, without limitation, fibroblast  
25   mitogenic activity ("FMA"), endothelial cell chemotaxis activity ("ECCA"), rabbit corneal assay activity ("RCAA") and keratinocyte cell chemotaxis activity ("KCCA"). Example 9 shows the correlation between B-TG and FMA, while examples 10-12 disclose assays defining the  
30   additional activities.

EXAMPLE 9

41 autologous platelet releasate samples were assayed for B-TG by the above B-TG immunoassay and for FMA by the following FMA protocol.

35

SET UP

1. Determine the number of microtiter plates necessary for FMA samples to

be tested. (One plate will accommodate 24 quadruplicate or 32 triplicate samples "including" necessary controls).

- 5                   2. Prepare approximately 20 ml per plate of Delbucco's Modified Eagle Medium (DMEM) containing 10% heat-inactivated calf serum (10% HI-CS). Prepare an additional 40 ml DMEM/10% HI-CS (used for preparing the cells).
- 10                  3. Thaw out appropriate number of tubes of 3T3 (A31) fibroblasts, which are stored frozen in liquid nitrogen, in a 37°C water bath. (Yield per tube will vary with frozen batch. Approximately 2,000,000 viable cells per microtiter plate will be needed.)
- 15                  4. Aseptically transfer cells to sterile 50 ml culture tube (12 ml and 15 ml can also serve this purpose, just cut back on the liquid) containing 20 ml (5-10 ml) DMEM/10% HI-CS. "Resuspend well" and centrifuge at 450xg (1400rpm in Mistral 3000i with shielded swing bucket rotors) for 10 minutes at R.T. Decant supernatant, resuspend cellular pellet in 10 ml DMEM/10% HI-CS, transferring it to a sterile 12 ml culture tube. Repeat centrifugation.
- 20                  5. Resuspend cellular pellet in approximately 2-5 ml DMEM/10% HI-CS. Perform cell count.
- 25                  6. Dilute the cells in DMEM/10% HI-CS to obtain a concentration of 200,000 cells per ml. (For each plate 10-11 mls are needed).
- 30                  7. Add 100ul per well to 96 well microtiter dish using an 8 or 12 multi-channel pipettor and sterile boat reservoir. Be sure to draw suspension in and out of the pipettor at least once per row to maintain cell-suspension adequately).
- 35                  8. Add 100ul DMEM/10% HI-CS to each well (for a combined total of 200 ul liquid per well).
- 40                  9. Label plates with cell line and date of plate preparation. Incubate plates at 37°C, 5% CO<sub>2</sub> for 3 days or until fibroblasts are confluent.
- 45

5

- 10

- 15

- 20

- 25

- 30

7. Retain excess 0.8% HI-CS for making dilutions.

Six hours after changing the media from 10% to 0.8% HI-CS, the cells are ready to be stimulated.

- 35

- 40

3. The next 3 or 4 wells (working

25

horizontally) receive both 20 ul undiluted HI-CS and 30 ul 0.8% HI-CS per well. (Final dilution therefore = 10% HI-CS).

- 5                   4. The next 3 or 4 wells receive 50 ul of Platelet Buffer control (10 ml Platelet Buffer + 50 ul thrombin).
5. Add 50 ul of the test/control samples.
- 10                 6. Incubate the plates 37°C at 5% CO<sub>2</sub> for 18 hours. (Consistency in timing is important).

#### RADIOACTIVE LABELING

                  Eighteen hours after stimulation with test and control samples, the FMA microtiter plates are labeled with radioactive thymidine to demonstrate mitogenic activity.

- 15                 1. Line work surface area with disposable paper liner to contain any accidental spills while working with the radionuclides. Protective gloves should be worn.
- 20                 2. Prepare a 10 uCi [3H]-thymidine/ml DMEM solution as follows: Sterile transfer 0.5 cc [3H]-thymidine (NEN cat no. NET-027, 6.7 Ci/mmol, 1mCi/ml) to 49.5 ml DMEM (a 1/100 dilution).
- 25                 3. Add 50 ul of [3H]-thymidine/DMEM solution to each well. Store leftover radioactive solution in the refrigerator for next time.
- 30                 4. Properly discard pipette tips, gloves, dispensing container holding the aliquot of labeled media, and paper liner in the radioactive waste.
- 35                 5. Label plates as radioactive and incubate in a tray to contain any spills at 37°C, 5% CO<sub>2</sub> for 6 hours.

#### HARVESTING

- 40                 1. Carefully aspirate off radioactive culture media using a NUNC immuno wash. Be sure to use "raise pins" provided, to prevent contact of aspiration prongs with the cells.

2. Wash cells by adding 200 ul PBS with multi-channel pipettor. Aspirate with NUNC immuno wash.
- 5 3. Add 200 ul 0.25% trypsin/HBSS (Ca, Mg free) to each well. Incubate at 37°C, 5% CO<sub>2</sub> for 30 minutes.
4. Harvest plate onto glass filter paper using the Skatron Combi Cell Harvester.
- 10 5. Immediately transfer wet filter paper discs into scintillation vials (Packard Pico Pro Vials) using the Skatron Filter Transfer apparatus.
6. Allow filter discs to dry overnight or in drying oven for 1-2 hours.

15

SCINTILLATION PREPARATION

1. Add 4 ml scintillation cocktail (Beckman Ready-Safe) to each vial.
- 20 2. Vials are capped tightly and shaken back and forth vigorously a few times to expose filter completely to the cocktail and dislodge potential air bubbles.

COUNTING

- 25 1. Place vials into the Beckman LS1701 green racks in order from left to right.
2. Place "program rack" into counter first, consisting of an empty green rack with ONE vial in the 18th position, telling the machine to use Program No. 1.
- 30 3. Program No. 1 is programmed as follows:
- Replicates: 3
  - Count time: 2 minutes
  - H#: No
  - Sample Repeat: 1

35 - Data calc: CPM

  - SCR: Yes
  - RCM: Yes
  - Vial size: Mini
  - Count Blank: No
- 40 4. Place remaining racks into counter working "back to front" on the right

side first, then "front to back" on the left side. Always end with the RED stop rack.

- 5                   5. Push both "RESET" buttons at the same time.
6. When RESET is complete, and the printer has been checked for enough paper, press the START button and replace the cover.
- 10                  7. Monitor the initial print-out to confirm accuracy of program being used.

Units of 1/ED-50 represents the dilution of the platelet releasate sample which results in a 50% stimulation of mitogenic activity in fibroblast 3T3 cells. For example, if a 0.25 or 1:4 dilution of the sample gave 50% stimulation, the 1/ED-50 would be 4 units. Similarly, a 1:8 dilution would give an 1/ED-50 of 8 units.

20                  The amount of B-TG in the platelet releasate sample correlated to the FMA activity as follows:

Variables:	B-TG VS FMA
Sample Size:	41
Spearman R:	0.7674
T-Value:	6.1927
25           2-Tail P:	< 0.0001

FIG. 6 shows a representative plot of the data.

#### EXAMPLE 11

ECCA activity may be defined by the following protocol.

#### 30                   PREPARATION OF CELLS

1. Grow Rabbit Wound Capillary Endothelial (RWCE) to 60-85% "confluent" on 3-4 Primaria (Falcon #3824) 75 cm<sup>2</sup> flasks.
- 35           2. Approximately 20-24 hours before chemotaxis, remove the media and rinse flasks twice (2X) with HBSS (Ca/Mg free, 6 ml/flask).
- 40           3. Remove last HBSS wash and add 12-15 ml (be consistent) of 0.2% lactalbumin in Media 199 to each flask. (This

provides minimal nutrients and reduces serum induced stimulation, so the cells are ready to respond to the attractants.) Record time of media change on flask.

- 5                   4. The following day, prepare the following:
  - a) 50 -100 ml 0.2% lactalbumin in M199 (LA-M199)
  - b) 20-30 ml (5 ml/75cm<sup>2</sup> flask) Enzyme Cocktail No. 2 (EC2-1X) by diluting 1 ml EC2(10X) in 9 ml HBSS.
- 10               5. Remove 0.2% LA-M199 and rinse flask with 6-10 ml HBSS. Immediately add 5 ml EC2(1X) and incubate for exactly 14 minutes at R.T.
- 15               6. Pool EC2 from flasks into 50 ml polypropylene tube(s) containing at least 2 ml/flask 0.2% LA-M199, to help deactivate the enzymes. Immediately add 5 ml 0.2% LA-M199 to each flask.
- 20               7. Gently scrape off cells from bottom with a sterile cell scraper (American Scientific Products Cat. # T4206-1).
- 25               8. Add cells/media to the EC2 pool. For a final rinse, add 10 ml 0.2% LA-M199 to one flask. Transfer rinse wash from flask to flask, then pool with cells.
- 30               9. If final volume exceeds 40 ml, divide cells into two tubes for centrifugation. Centrifuge cells at 1400 rpm (approx. 450g on Mistral 3000 centrifuge) for 10 minutes at R.T.
- 35               10. Discard supernatant by quickly pouring off. Resuspend pellet(s) in 8 ml total 0.2% LA-M199 (pool if divided) and transfer to a 15ml centrifuge tube. Wash tubes with an additional 2 ml media and add to resuspended cells. Centrifuge at 1400 rpm for 10 minutes at R.T.
- 40               11. Resuspend cells in 2-5 ml 0.2% LA-M199 (adjust volume to size of pellet and estimated cell yield: to avoid recentrifugation) for counting.



## 12. Count cells:

- 5                   a) Add 30 ul cell suspension to 30 ul Trypan Blue.
- b) Load both sides of heamocytometer.
- c) Under 10X mag., count cells in eight lmm squares. Record number of viable (blue) cells. Do not count cells of abnormal size or shape.
- 10                  d) Multiply<sub>3</sub> viable cell count by  $2.5 \times 10^3$  to = cells per ml.

13. Adjust cell concentration to  $0.75 \times 10^6$  cells/ml LA-M199 (i.e., 33,750 cells per/well in 45 ul). Approximately 2.25 ml/chamber is needed.

- a) Example: There are 3 ml of  $1.5 \times 10^6$  cells per ml. The final adjusted volume should be:

$$\frac{(3 \text{ ml}) (1.5 \times 10^6 \text{ cells/ml})}{(0.75 \times 10^6 \text{ cells/ml})} = 5.95 \text{ ml}$$

Therefore, add 2.95 ml 0.2% LA-M199 to cells to obtain a final concentration of  $0.75 \times 10^6$ . In short, prepare 45 wells worth of cells/chamber at a concentration of 33,750 cells/45 ul 0.2% LA-M199/well.

## \* Suggested Volumes

	Flask size (cm <sup>2</sup> ) (ml)	0.2% LA-M199 feed media (ml)	HBSS wash (ml)	EC2(1X)
30	25	5	5	3
	75	15	6-10	5
35	150	30	10-15	10

PREPARATION OF FILTERS

1. Prepare 20 ml of 1 ug Fibronectin (Sigma #F4759)/1 ml HBSS (FN/HBSS) from frozen stock. Use polypropylene tips and tubes only for its preparation. (Example: Frozen stock = 1 ng/ml dH<sub>2</sub>O-HBSS. Therefore dilute 200 ul stock in 19.8 ml HBSS). Store on ice until

use.

2. Use Nuclepore polypropylene filters (8.0  $\mu$ m pores, PVPF, from Neuro Probe Inc., 301-229-8598), one per chamber.
  - 5 3. Cut the upper left-hand corner off the shiny side of the filter to provide filter orientation. Use tweezers to handle the filter, (never fingers) and only at the ends.
  - 10 4. Place 3-4 ml FN/HBSS in the center of a sterile petri dish. Lay filter on top of the FN/HBSS, shiny side down, allowing to spread beneath the filter; do not allow any FN/HBSS on the top of the filter.
  - 15 5. Cover petri dish and allow to stand at R.T. for 30 minutes.
  - 20 6. Carefully pour off FN/HBSS (by tilting majority to one side slowly, allowing filter to stick to plate, then pouring off completely), lift up filter, place 3-4 ml fresh FN/HBSS in center and repeat procedure on other side of filter (dull side down).
  - 25 7. Coating is now complete and filters can be used.
- \* Note: a) For consistency, use filter immediately, by timing the filling of the bottom chamber to coincide with the completion of coating the second side of the filter.
- b) When preparing two filters, it is recommended to stagger their coating times by 15 minutes to allow for the filling of one chamber before having to fill the second, to alleviate having to rush to fill both at the same time.

#### PREPARATION OF CHAMBERS

1. Remove Neuro Probe 48 well chemotaxis chambers from distilled water ( $\text{dH}_2\text{O}$ ) storage bath. Rinse thoroughly with clean  $\text{dH}_2\text{O}$ . Dry top piece and gasket

with tissue, and blow dry bottom piece with clean nitrogen gas.

- 5                   2. Prepare test samples to load bottom of chamber. Each well holds approximately 26.0 - 26.5 ul) solution.

3. Add (approximately 26.1 - 26.5 ul) samples to the wells in the lower chamber.

10                   \* Note: a) To produce a desired "positive meniscus", slight "topping off" with fluid may be necessary. This helps to counteract drying which occurs while filling the rest of the chamber. Avoid creating bubbles.

15                                   b) Use a positive displacement pipette for best consistency. Since the first and last columns (A,L) of four wells on either chamber end are not utilized for chemotaxis, fill them with HBSS. Consequently, these same rows in the upper chamber are also filled with HBSS and not cells.

20

- 25                   4. When the filters are ready, pour off FN/HBSS (as previously described). Carefully lift the filter off the petri dish; do not let either side scrape against the edge of the dish. By lifting filter slowly, the residual FN/HBSS on the filter is minimized. DO NOT drop or carelessly touch the filter at this point.
- 30

- 35                   5. While holding both ends of the filter with forceps, lower the center of the filter onto the center of the chamber, then evenly cover bottom wells. The filter should be shiny side up!

40                   \* Note: Always orient chamber/filter consistently; i.e., always keep chamber trademark and cut corner on filter in the upper left-hand corner.

- 45                   6. Only if necessary, adjust the filter slightly to properly align.

7. Lay the gasket just above the filter,

but not touching.

- 5                   8. Place the upper half of the chamber on top of gasket and push down both together. Hold them down tightly while securing the retaining screws.
- 10                  9. Once secure, pick up chamber and look through wells for any bubbles that may have formed underneath filter; record these, as they can interfere with chemotaxis.
10. Add 45 ul HBSS to the four wells on both ends (A,L).
- 15                  11. Next add 45 ul of cell suspension (i.e.,  $0.75 \times 10^6$  cells per ml) to the remaining wells.
- 20                  \* Note: Add cells with pipette tip at an angle to prevent trapping air in the bottom. If a bubble forms, carefully withdraw liquid and refill. After filling, all wells should look uniform. If not, suspect trapped air and redo.
- 25                  12. Place the chamber in a glass or polypropylene tray, add a water soaked gauze square (increasing humidity and preventing evaporation), and loosely cover with aluminum foil.
13. Incubate for 4 hours at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ .

REMOVAL AND WIPING OF FILTER

- 30                  1. Etch a glass slide at one end with date and chamber number. Clean well with alcohol prep and dry.
2. Remove retaining nuts while holding down top plate.
- 35                  3. Orient chamber with trademark in upper left-hand corner, over a paper towel.
4. Invert entire chamber (along horizontal axis) onto paper towel.
- 40                  5. Push down on the four corners of the top plate so it stays parallel to the bottom plate as it drops. The filter should be stuck to the gasket.

6. Remove the bottom plate and immerse immediately in Tergazyme solution (1/4 teaspoon Tergazyme/1000 ml  $\text{dH}_2\text{O}$ ).
- 5 7. The "migrated cells" are now facing up. DO NOT disturb this face of the filter from here on.
- 10 8. Catch the very right-hand edge of the filter with forceps, lift edge to loosen, then pull filter slightly to the right so the end just hangs over the edge.
- 15 9. Clamp this end with the plastic clip and lift the filter off the gasket. Quickly apply the second plastic clip to the other end. Place top chamber piece immediately in Tergazyme.
- 20 10. Keeping the cell side up (ALWAYS), wet the non-migrated side in PBS. Do not let PBS wet the "migrated cells" side.
- 25 11. Holding the filter taut, draw the non-migrated side against the wiper blade (from one end to the next in one direction only.)
- 30 12. Repeat this procedure 4-5 times. Minimize time between wetting and wiping to prevent non-migrated cells from drying/sticking and causing incomplete removal. ALWAYS dry wiper blade before each successive wipe.
- 35 13. Place filter on appropriate etched slide, with cut corner on same end as etching, but on opposite side. Allow to dry overnight.
14. Rinse off chamber pieces sitting in Tergazyme with  $\text{dH}_2\text{O}$  and store in fresh  $\text{dH}_2\text{O}$ , covered, until chambers are to be cleaned.

#### STAINING OF FILTER

40 Set up the Densitometer (LKB) so the filter can be read immediately after staining.

1. Place small black clip on the end of the dried filter/slide with the cut corner.

2. Stain in LeukoStat (Fisher brand) by dipping in each of three solutions, in order, 5 times for 5 seconds each time. Dab off excess stain on a paper towel or gauze between solutions.
3. Allow filter to sit in third stain for 30 seconds extra after the 5 dips.
4. Rinse filter in  $\text{dH}_2\text{O}$  (use two changes of  $\text{dH}_2\text{O}$ ). Dab excess  $\text{dH}_2\text{O}$  off.
5. Place another clean glass slide (unmarked) directly over the filter, and press together carefully yet firmly, forcing out most of the air bubbles.
6. Read on the Densitometer.

#### DENSITOMETRY READING OF STAINED FILTER

1. Allow Densitometer (LKB) to warm up for 10-20 minutes.
2. Place stained, wet slide on reading table and orient to proper coordinates as follows:

	<u>Column</u>	<u>X Position</u>	<u>"Track"</u>
	B	113.6	1
	C	119.6	2
	D	127.0	3
25	E	132.6	4
	F	138.6	5
	G	146.6	6
	H	152.6	7
	I	158.0	8
30	J	166.0	9
	K	171.6	10

Additional densitometer settings:

- a) Smoothing: 3
- b) x-width: 4
- c) y-start: 19
- d) y-stop: 43

3. Line up slide. Check column positions.
4. Clip slide down without moving it.
5. Check "Y" coordinates on various rows.

6. Send ruler "home". "Ecs".
7. Close lid.
8. "Enter" (on computer), then "6" (or "Run") on Densitometer.
- 5 9. Calculate area of peaks from Densitometer using LKB's "GSXL" program.

#### CLEANING CHAMBERS

The following procedure is used to remove residual proteins, etc., from the chemotaxis chambers and gaskets. (Source: Terri Superdock, 118:61, 2/21/89).

- 15 1. Rinse dirty gaskets and chambers well with deionized water. Place chambers with corresponding gaskets in a 1 liter plastic beaker (2 sets/beaker).
2. Heat 0.75% Tergazyme solution (7.5 gm Tergazyme/1 liter  $\text{dH}_2\text{O}$ ; 500-750 ml/2 chambers) to  $50^\circ\text{C}$ . DO NOT EXCEED  $50^\circ\text{C}$ .
- 20 3. Cover chambers and gaskets with  $50^\circ\text{C}$  Tergazyme.
4. Place beaker(s) in a  $50^\circ\text{C}$  waterbath. Cover bath and incubate for 2 hours.

\* For gasket cleaning see steps 10 and 11.

- 25 5. Remove chambers only and rinse well with  $\text{dH}_2\text{O}$ . Place chambers in a 1 liter plastic beaker (1000 ml); 2-3 chambers/beaker.
6. Cover chambers with R.T. 1 M NaOH (600-700 ml/beaker). Cover beaker with tin foil.
- 30 7. Incubate beaker(s) in a  $50^\circ\text{C}$  covered waterbath for 30 minutes.
8. Rinse chambers very well with  $\text{dH}_2\text{O}$ . Place chambers and a large stir bar in deep plastic tub. Strategically orient chambers (tops and bottoms) so as not to interfere with the spinning stir bar. Put tub on a magnetic stirrer near a sink.
- 35

- 5 9. Fill tub with  $\text{dH}_2\text{O}$ , letting it run continuously for 2 hours. Make sure the water is circulating adequately, and a syphoning system is placed in the tub leading to the sink to prevent overflow.
- 10 10. Place gaskets into one beaker with 0.75% Tergazyme and sonicate for 30 minutes.
- 10 11. Rinse well with  $\text{dH}_2\text{O}$  and place gaskets in 1 liter  $\text{dH}_2\text{O}$ . Sonicate for 2 hours, changing the water every 30 minutes.
- 15 12. Assemble chambers and gaskets (lightly tighten with screws) and place in flat pan (polypropylene) filled with fresh  $\text{dH}_2\text{O}$ . Cover with aluminum foil, and change water once a week.
- 20 13. Rinse chambers and gaskets well with fresh  $\text{dH}_2\text{O}$  before use.

#### EXAMPLE 12

KCCA activity may be defined by the following protocol.

#### PREPARATION OF CELLS

- 25 1. Obtain an EpiPack containing proliferating cells in T-25 Flask of Normal Human Epidermal Keratinocyte (NHEK), 500ml bottles of KGM (Keratinocyte Growth Medium Supplemental and Serum Free), 30 500ml bottle KBM (Keratinocyte Basal Medium) and subculture reagents consisting of HEPES Buffered Saline Solution Trypsin [(0.025% W/V)/EDTA (0.01% W/V)] solution Trypsin neutralizing solution from Clonetics Corporation, San Diego, 35 California.
2. Upon arrival, unpack and incubate sealed T-25 flask at  $37^\circ\text{C}$  5%  $\text{CO}_2$  to equilibrate temp.
3. Warm 5ml KGM in sterile container.
- 40 4. Wipe T-25 flask thoroughly with 70% isopropyl alcohol under sterile field (Biohood).
5. Remove medium; discard in container



containing a little bleach and replace with 5ml warmed KGM. Leave cap screwed on but not tightly close.

- 5                   6. Return to incubator @ 37°C, humidified with 5% CO<sub>2</sub> for 24 - 48 hours for subculturing. Do not allow cell culture to become confluent.

PASSING CELLS OFF T-25 FLASK

- 10               1. Under biohood, remove culture medium (discard in container with a little bleach) and wash cells with 2ml HEPES buffered solution.
2. Remove HEPES add 2ml Trypsin/EDTA Solution, let sit for 2 minutes.
- 15               3. Remove Trypsin/EDTA and place in sterile centrifuge tube containing 2ml Trypsin Neutralizing Solution. Tighten cap on flask and look under scope.
- 20               4. Look for cells detaching and rounded up. After 3 additional min., hit flask against palm of hand once on one side and once on other side. Look under scope for cells floating. Do not go over 4 min. total since trypsinizing.
- 25               5. Under biohood, immediately add 2ml Trypsin Neutralizing Solution and wash. Transfer cells to sterile centrifuge tube (see #3), rinse flask with another 2ml of Trypsin Neutralizing Solution
- 30               and place in centrifuge tube.
6. Check flask under scope; tighten cap to see if any cells are left.
7. If there are a lot of cells left, repeat entire procedure from step 1 and add to centrifuge tube. If there are no cells or very small amount, proceed to centrifuge tube.
- 35               8. Centrifuge cells at 220 x G @ 25°C for 10 min., discard supernate.
- 40               9. Resuspend cell pellet with warm 5ml KGM and count with hemocytometer.
10. Inoculate new flask at desire density.

PASSING CELLS OFF T-75 flask (- 70 - 80% CONFLUENT):

1. Follow same procedure for T-25 flask except use following amount:
  - a. 5ml HEPES buffered solution
  - b. 7ml Trypsin/EDTA solution
  - c. Wash cells off flask with 7ml Trypsin Neutralizing Solution, transfer to blue max tube.
  - d. Wash flask again with 3ml Trypsin Neutralizing Solution and place in blue max tube.
2. Centrifuge @ 220 X G @ 25°C for 10 min. Discard supernate.
3. Resuspend cell pellet with 5ml KBM and place in centrifuge tube.
4. Centrifuge, discard supernate and resuspend with 2ml KBM and count with hemocytometer.
5. Run chemotaxis assay and set up new densities.

- NOTE:
1. For chemotaxis purposes, resuspend pellet with KBM.
  2. For passing cells to set up new cell density only, use KGM.
  3. Feed cells with fresh KGM M, W, F.

To T-75 flask = 15ml KGM

T-25 flask = 5ml KGM

PREPARATION OF CELL FOR CHEMOTAXIS

1. Trypsinize and spin down cells according to the procedure outlined under passing cells off T-75 flask.
2. Resuspend cells in KBM and count with a hemacytometer. Total cells = (Ave. count) x (ml KBM) x (trypan blue dilution) x  $(1 \times 10^4)$ .
3. Make final cell dilution of  $5.56 \times 10^5$  cells/ml (or 25,000 cells/45 ul).

Keep cells on ice until needed.

PREPARATION OF FILTER

- 5 1. Prepare 20ml of 5ug/ml Fibronectin solution (Sigma #F4759) from frozen stock. Use polypropylene sterile tip and tubes for its preparation. (Example: Frozen stock = 0.1mg/ml dH<sub>2</sub>O - HBSS. Therefore dilute 1,000ul stock in 19.0ml HBSS). Store on ice until used.
  - 10 2. Use Nucleopore polypropylene filters (8.0 um pores, PVPP, from Neuro Probe Inc., 301-229-8598), one per chamber.
  - 15 3. Cut the upper left-hand corner off the shiny side of the filter to provide filter orientation. Use tweezers to handle the filter, (never fingers) and only at the ends.
  - 20 4. Place 3-4 ml FN/HBSS in the center of a sterile petri dish. Lay filter on top of the FN/HBSS, shiny side down, allowing to spread beneath the filter; do not allow any FN/HBSS on the top of the filter.
  - 25 5. Cover petri dish and allow to stand at R.T. for 30 minutes.
  - 30 6. Carefully pour off FN/HBSS (by tilting majority to one side slowly, allowing filter to stick to plate, then pouring off completely), lift up filter, place 3-4 ml fresh FN/HBSS in center and repeat procedure on other side of filter (dull side down).
  - 35 7. Coating is now complete and filters can be used.
- 35 \* NOTE: a) For consistency, use filter immediately, by timing the filling of the bottom chamber to coincide with the completion of coating the second side
- 40 of the filter.
- b) When preparing two filters, it is recommended to stagger their coating times by 15 minutes to allow for the filling of one chamber before having to
- 45

fill the second, to alleviate  
having to rush to fill both  
at the same time.

## PREPARATION OF CHAMBERS

- 5 1. Remove Neuro Probe 48 well chemotaxis chambers from distilled water (dH<sub>2</sub>O) storage bath. Rinse thoroughly with clean dH<sub>2</sub>O. Dry top piece and gasket with tissue, and blow dry bottom piece with clean nitrogen gas.
- 10 2. Prepare test samples to load bottom of chamber. Each well holds approximately 26.0 - 26.5 ul) solution.
- 15 3. Add (approximately 26.1 - 26.5 ul) samples to the wells in the lower chamber.
- \* NOTE: a) To produce a desired "positive meniscus", slight "topping off" with fluid may be necessary. This helps to counteract drying which occurs while filling the rest of the chamber. Avoid creating bubbles.
- 20 b) Use a positive displacement pipette for best consistency. Since the first and last columns (A,L) of four wells on either chamber end are not utilized for chemotaxis, fill them with HBSS. Consequently, these same rows in the upper chamber are also filled with HBSS and not cells.
- 25 4. When the filters are ready, pour off FN/HBSS (as previously described). Carefully lift the filter off the petri dish; do not let either side scrape against the edge of the dish. By lifting filter slowly, the residual FN/HBSS on the filter is minimized. DO NOT drop or carelessly touch the filter at this point.
- 30 5. While holding both ends of the filter with forceps, lower the center of the filter onto the center of the chamber, then evenly cover bottom wells. The filter should be shiny side up!
- 35 46

\* NOTE: Always orient chamber/filter consistently; i.e., always keep chamber trademark and cut corner on filter in the upper left-hand corner.

5

6. Only if necessary, adjust the filter slightly to properly align.

7. Lay the gasket just above the filter, but not touching.

10

8. Place the upper half of the chamber on top of gasket and push down both together. Hold them down tightly while securing the retaining screws.

15

9. Once secure, pick up chamber and look through wells for any bubbles that may have formed underneath filter; record these, as they can interfere with chemotaxis.

20

10. Add 45 ul HBSS to the four wells on both ends (A,L).

11. Next add 45 ul of cell suspension to the remaining wells.

25

\* NOTE: Add cells with pipette tip at an angle to prevent trapping air in the bottom. If a bubble forms, carefully withdraw liquid and refill. After filling, all wells should look uniform. If not, suspect trapped air and redo.

30

12. Place the chamber in a glass or polypropylene tray, add a water soaked gauze square (increasing humidity and preventing evaporation), and loosely cover with aluminum foil.

35

13. Incubate for 18 hours at 37°C, 5% CO<sub>2</sub>.

#### REMOVAL AND WIPING OF FILTER

40

1. Etch a glass slide at one end with date and chamber number. Clean well with alcohol prep and dry.

2. Remove retaining nuts while holding down top plate.

3. Orient chamber with trademark in upper

left-hand corner, over a paper towel.

4. Invert entire chamber (along horizontal axis) onto paper towel.
5. Push down on the four corners of the top plate so it stays parallel to the bottom plate as it drops. The filter should be stuck to the gasket.
10. Remove the bottom plate and immerse immediately in Tergazyme solution (1/4 teaspoon Tergazyme/1000 ml  $\text{dH}_2\text{O}$ ).
7. The "migrated cells" are now facing up. DO NOT disturb this face of the filter from here on.
15. Catch the very right-hand edge of the filter with forceps, lift edge to loosen, then pull filter slightly to the right so the end just hangs over the edge.
20. Clamp this end with the plastic clip and lift the filter off the gasket. Quickly apply the second plastic clip to the other end. Place top chamber piece immediately in Tergazyme.
25. Keeping the cell side up (ALWAYS), wet the non-migrated side in PBS. Do not let PBS wet the "migrated cells" side.
30. Holding the filter taut, draw the non-migrated side against the wiper blade (from one end to the next in one direction only).
35. Repeat this procedure 4-5 times. Minimize time between wetting and wiping to prevent non-migrated cells from drying/sticking and causing incomplete removal. ALWAYS dry wiper blade before each successive wipe.
40. Place filter on appropriate etched slide, with cut corner on same end as etching, but on opposite side. Allow to dry overnight.
14. Rinse off chamber pieces sitting in Tergazyme with  $\text{dH}_2\text{O}$  and store in fresh  $\text{dH}_2\text{O}$ , covered, until chambers are to be cleaned.

STAINING OF FILTER

Set up the Densitometer (LKB) so the filter can be read immediately after staining.

- 5 1. Place small black clip on the end of the dried filter/slide with the cut corner.
- 10 2. Stain in LeukoStat (Fisher brand) by dipping in each of three solutions, in order, 5 times for 5 seconds each time. Dab off excess stain on a paper towel or gauze between solutions.
- 15 3. Allow filter to sit in third stain for 30 seconds extra after the 5 dips.
4. Rinse filter in  $\text{dH}_2\text{O}$  (use two changes of  $\text{dH}_2\text{O}$ ). Dab excess  $\text{dH}_2\text{O}$  off.
5. Place another clean glass slide (unmarked) directly over the filter, and press together carefully yet firmly, forcing out most of the air bubbles.
- 20 6. Read on the Densitometer.

DENSITOMETRY READING OF STAINED FILTER

1. Allow Densitometer (LKB) to warm up for 10-20 minutes.
- 25 2. Place stained, wet slide on reading table and orient to proper coordinates as follows:

	<u>Column</u>	<u>X Position</u>	<u>"Track"</u>
	B	113.6	1
	C	119.6	2
30	D	127.0	3
	E	132.6	4
	F	138.6	5
	G	146.6	6
	H	152.6	7
35	I	158.0	8
	J	166.0	9
	K	171.6	10

Additional densitometer settings:

- a) Smoothing: 3
- 40 b) x-width: 4

- c) y-start: 19
- d) y-stop: 43
- 3. Line up slide. Check column positions.
- 4. Clip slide down without moving it.
- 5. Check "Y" coordinates on various rows.
- 6. Send ruler "home". "Ecs".
- 7. Close lid.
- 8. "Enter" (on computer), then "6" (or "Run") on Densitometer.
- 9. Calculate area of peaks from Densitometer using LKB's "GSXL" program.

#### CLEANING CHAMBERS

The following procedure is used to remove residual proteins, etc., from the chemotaxis chambers and gaskets. (Source: Terri Superdock, 118:61, 2/21/89).

- 1. Rinse dirty gaskets and chambers well with deionized water. Place chambers with corresponding gaskets in a 1 liter plastic beaker (2 sets/beaker).
- 2. Heat 0.75% Tergazyme solution (7.5 gm Tergazyme/1 liter  $\text{dH}_2\text{O}$ ; 500-750 ml/2 chambers) to  $50^\circ\text{C}$ . DO NOT EXCEED  $50^\circ\text{C}$ .
- 3. Cover chambers and gaskets with  $50^\circ\text{C}$  Tergazyme.
- 4. Place beaker(s) in a  $50^\circ\text{C}$  waterbath. Cover bath and incubate for 2 hours.  
\* For gasket cleaning see steps 10 and 11.
- 5. Remove chambers only and rinse well with  $\text{dH}_2\text{O}$ . Place chambers in a 1 liter plastic beaker (1000 ml); 2-3 chambers/beaker.
- 6. Cover chambers with R.T. 1 M NaOH (600-700 ml/beaker). Cover beaker with tin foil.
- 7. Incubate beaker(s) in a  $50^\circ\text{C}$  covered waterbath for 30 minutes.
- 8. Rinse chambers very well with  $\text{dH}_2\text{O}$ .



- 5 Place chambers and a large stir bar in deep plastic tub. Strategically orient chambers (tops and bottoms) so as not to interfere with the spinning stir bar. Put tub on a magnetic stirrer near a sink.
- 10 9. Fill tub with  $\text{dH}_2\text{O}$ , letting it run continuously for 2 hours. Make sure the water is circulating adequately, and a syphoning system is placed in the tub leading to the sink to prevent overflow.
- 15 10. Place gaskets into one beaker with 0.75% Tergazyme and sonicate for 30 minutes.
- 20 11. Rinse well with  $\text{dH}_2\text{O}$  and place gaskets in 1 liter  $\text{dH}_2\text{O}$ . Sonicate for 2 hours, changing the water every 30 minutes.
- 25 12. Assemble chambers and gaskets (lightly tighten with screws) and place in flat pan (polypropylene) filled with fresh  $\text{dH}_2\text{O}$ . Cover with aluminum foil, and change water once a week.
13. Rinse chambers and gaskets well with fresh  $\text{dH}_2\text{O}$  before use.

#### EXAMPLE 13

RCAA activity may be defined by the following protocol.

- 30 2-4 polymer pellets are made for each sample to be tested for angiogenic activity. A solution of 10% v/v polymer, available as Hydron<sup>®</sup> polymer, type NCC, cell culture grade, (available commercially from HydroMed Sciences, New Brunswick, NJ 08901), 1% v/v polyethyleneglycol in 70% v/v ethanol should
- 35 be prepared (hereinafter polymer solution). Polymer solution is mixed 1:1 v/v with test sample. A piece of plastic autoclave bag is taped onto a flat surface, making sure it is taut. The surface is then wiped off with an alcohol prep, and allowed to dry. 20 ul
- 40 of the 1:1 mixture is dropped onto the plastic. The polymer pellets are then dried under vacuum for

approximately 2 hours, or until dry.

The corneal implant assay is conducted on a 4-6 lb. New Zealand White Rabbit. Anesthetic is prepared by mixing 1:1 v/v Ketamine hydrochloride 100mg/ml, commercially available as Ketaset<sup>®</sup> from Veterinary Products, Bristol Laboratories, Syracuse, NY 13201, and acepromazine maleate 10mg/ml, commercially available as Promace<sup>®</sup> from Aveco Co., Inc., Fort Dodge, IA 50501, in the same syringe. 4-5cc is used for each rabbit. Anesthetic is injected into the gluteus maximum or gastrocnemius using a 23 gauge needle, gently rubbing the area after injection. The rabbit is properly anesthetized when it cannot resist being rolled onto its back, usually in 10-15 minutes.

The rabbit is placed on a sterile drape. 3-5 drops of proparacaine hydrochloride 0.5%, commercially available as Ophthetic<sup>®</sup> from Allergan Pharmaceuticals, Inc., Irvine, CA 92713, are put in each eye to numb the area. The anesthetic solution is used as needed throughout the procedure whenever the eye becomes dry.

The eye is brought out of the socket using petite point tissue forceps. The forceps are slowly worked towards the inner corner of the eye and a bit of tissue is clamped to ensure the eye remains in this position while working, taking care not to clamp the optic nerve.

A scapel, Beaver eye blade No. 5210, commercially available from Beaver Surgical Products, Waltham, MA 02154, is gently drawn across the apex of the cornea, making an incision approximately 3.0 mm long. It is possible to puncture the cornea which will cause the aqueous humor to seep through. If this should occur, the animal must be sacrificed.

With a Elschmig cyclodialysis spatula, 1mm wide, 10mm long commercially available from U.

Mueller, Chicago, IL 60648, product #OP-2040, a canal is gently made through the cornea towards the capillary bed, stopping approximately 2 mm from the capillary bed. A "pocket" is made for the polymer pellet by moving the tip of a probe side to side, taking care not to move the probe forward as the pellet should not be closer than 1 mm to the capillary bed. A polymer pellet is lifted off the plastic using forceps and placed on the eye at the point of incision. With a spatula, the pellet is pushed through the canal and into the pocket. Several drops of anesthetic solution is used to lubricate the area and make insertion of the pellet easier. The pellet must be concentrated in the pocket. Trapped air is pushed out from the pocket by drawing a spatula along the canal on the outside of the cornea.

The forceps are then unfastened. The eyelids are gently pulled up and out manually and the eye resumes its normal position. Three drops of anti-bacterial solution, commercially available as Neosporin<sup>®</sup> Ophthalmic Solution from Burroughs Wellcome Co., Research Triangle Park, NC 27709, are put into each eye to minimize the possibility of infection.

One rabbit is used for each sample to be tested (i.e., 2 pellets of same sample per rabbit, one in each eye).

Eyes are checked on days 3, 5 and 7 for any direct growth of capillaries towards the pellet and graded according to the method of Gimbrone et al., J. Natl. Cancer Inst. 52:413-427(1974), and Banda et al., U.S. Patent No. 4,503,038, both of which are incorporated in their entirety by reference. Pictures of eyes are taken on day 7 to record capillary growth. Accordingly, the invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. From the foregoing, it will be obvious to those skilled

in the art that various modifications in the above-described methods and techniques can be made without departing from the spirit or scope of the invention.

CLAIMS

1. A method of making a platelet releasate product comprising:
  - performing an assay on a platelet  
5        releasate sample, where the assay indicates  
         the amount of a component present in the  
         platelet releasate sample; and
  - forming a platelet releasate product  
10        containing a selected amount of platelet  
         releasate, said amount selected by comparing  
         the amount of the component in the platelet  
         releasate sample to a predetermined range  
         of amounts of the identical component to  
         be contained in a platelet releasate product.
- 15    2. A method of treatment of tissue comprising:
  - applying a platelet releasate product  
         topically to tissue, where said platelet  
         releasate product contains a selected amount  
         of platelet releasate, said amount selected  
20        by comparison of the amount of a component  
         in a platelet releasate sample to a  
         predetermined range of amounts of the identical  
         component to be contained in a platelet  
         releasate product.
- 25    3. The method of claim 1 or 2 wherein said platelet  
         releasate contained in said platelet releasate product  
         and said platelet releasate sample are obtained from  
         the same draw of platelets.
- 30    4. The method of claim 1 or 2 wherein said platelet  
         releasate contained in said platelet releasate product  
         and said platelet releasate sample are obtained from  
         different draws of platelets from the same animal  
         or human.
5. The method of claim 1 or 2 wherein said platelet



releasate contained in said platelet releasate product and said platelet releasate sample are obtained from a pool of platelets from draws of platelets from a group of animal or human donors.

- 5 6. The method of claim 1 or 2 wherein the component present in the platelet releasate sample is selected from the group consisting of Beta-thromboglobulin, platelet derived growth factor, platelet derived angiogenesis factor, platelet factor 4, basic fibroblast growth factor, acidic fibroblast growth factor, transforming growth factor alpha, transforming growth factor beta, platelet derived epidermal growth factor and fibronectin.
- 10 7. The method of claim 6 wherein the component present in the platelet releasate sample is Beta-thromboglobulin.
- 15 8. The method of claim 7 wherein the platelet releasate product contains Beta-thromboglobulin at a concentration of greater than about 25 nanograms per milliliter of platelet releasate product.
- 20 9. The method of claim 6 wherein the component present in the platelet releasate sample is platelet derived growth factor.
- 25 10. The method of claim 9 wherein the platelet releasate product contains platelet derived growth factor at a concentration greater than about 0.2 nanograms per milliliter of a platelet releasate product.
11. The method of claim 6 wherein the component present in the platelet releasate sample is platelet factor 4.
- 30 12. The method of claim 11 wherein the platelet releasate product contains platelet factor 4 at a concentration greater than about 10 nanograms per milliliter of a platelet releasate product.
13. The method of claim 6 wherein the component

present in the platelet releasate sample is platelet derived angiogenesis factor.

14. The method of claim 1 or 2 wherein the selected amount of platelet releasate is sufficient to cause  
5 substantial efficacy of treatment of tissue.

15. The method of claim 14 wherein said efficacy is at least equivalent to grade 2 functional assessment score.

16. The method of claim 2 wherein the tissue is  
10 mammalian tissue.

17. The method of claim 16 wherein the tissue is human tissue.

18. The method of claim 3 wherein said platelets are mammalian platelets.

19. The method of claim 18 wherein said platelets are human platelets.  
15

20. The method of claim 4 wherein said animal is a mammal.

21. The method of claim 5 wherein said animal is  
20 a mammal.

22. A method of making a platelet releasate product comprising:

performing an assay on a platelet releasate sample, where the assay indicates  
25 the amount of an activity of the platelet releasate sample; and

forming a platelet releasate product containing a selected amount of platelet releasate, said amount selected by comparing  
30 the amount of said activity of the platelet releasate sample to a predetermined range of amounts of the identical activity of platelet releasate product.

23. A method of treatment of tissue comprising:



5                   applying a platelet releasate product  
topically to tissue, where said platelet  
releasate product contains a selected amount  
of platelet releasate, said amount selected  
by comparison of the amount of an activity  
of a platelet releasate sample to a  
predetermined range of amounts of the identical  
activity of platelet releasate product.

10       24. The method of claim 22 or 23 wherein said platelet  
releasate contained in said platelet releasate product  
and said platelet releasate sample are obtained from  
the same draw of platelets.

15       25. The method of claim 22 or 23 wherein said platelet  
releasate contained in said platelet releasate product  
and said platelet releasate sample are obtained from  
different draws of platelets from the same animal  
or person.

20       26. The method of claim 22 or 23 wherein said platelet  
releasate contained in said platelet releasate product  
and said platelet releasate sample are obtained from  
a pool of platelets from draws of platelets from  
a group of animal or human donors.

25       27. The method of claim 22 or 23 wherein the activity  
of the platelet releasate sample is selected from  
the group consisting of fibroblast mitogenic activity,  
endothelial cell chemotaxis activity, rabbit corneal  
assay activity and keratinocyte cell chemotaxis activity.

30       28. The method of claim 27 wherein the activity  
of the platelet releasate sample is fibroblast mitogenic  
activity.

29. The method of claim 28 wherein the platelet  
releasate product has a fibroblast mitogenic activity  
greater than about 2.5 1/ED-50 units per milliliter  
of platelet releasate product.

35       30. The method of claim 23 wherein the tissue is

mammalian tissue.

31. The method of claim 30 wherein the tissue is human tissue.

32. The method of claim 24 wherein said platelets  
5 are mammalian platelets.

33. The method of claim 32 wherein said platelets are human platelets.

34. The method of claim 25 wherein said animal is a mammal.

10 35. The method of claim 26 wherein said animal is a mammal.

36. The method of claim 22 or 23 wherein the selected amount of platelet releasate is sufficient to cause substantial efficacy of treatment of tissue.

15 37. The method of claim 36 wherein said efficacy is at least equivalent to grade 2 functional assessment score.

38. A platelet releasate product comprising:

- 20 (i) a selected amount of platelet releasate, said amount selected by comparison of the amount of a component in a platelet releasate sample to a predetermined range of amounts of the identical component to be contained in platelet releasate product; and
- 25 (ii) a pharmaceutically acceptable carrier or diluent for said platelet releasate.

39. The method of claim 38 wherein said composition is substantially free of blood or plasma contaminants and platelet ghosts or other material contained in  
30 platelets but not released by said platelets.

40. A platelet releasate product comprising:

- (i) a selected amount of platelet releasate, said amount selected by a comparison of the amount of an activity of a platelet

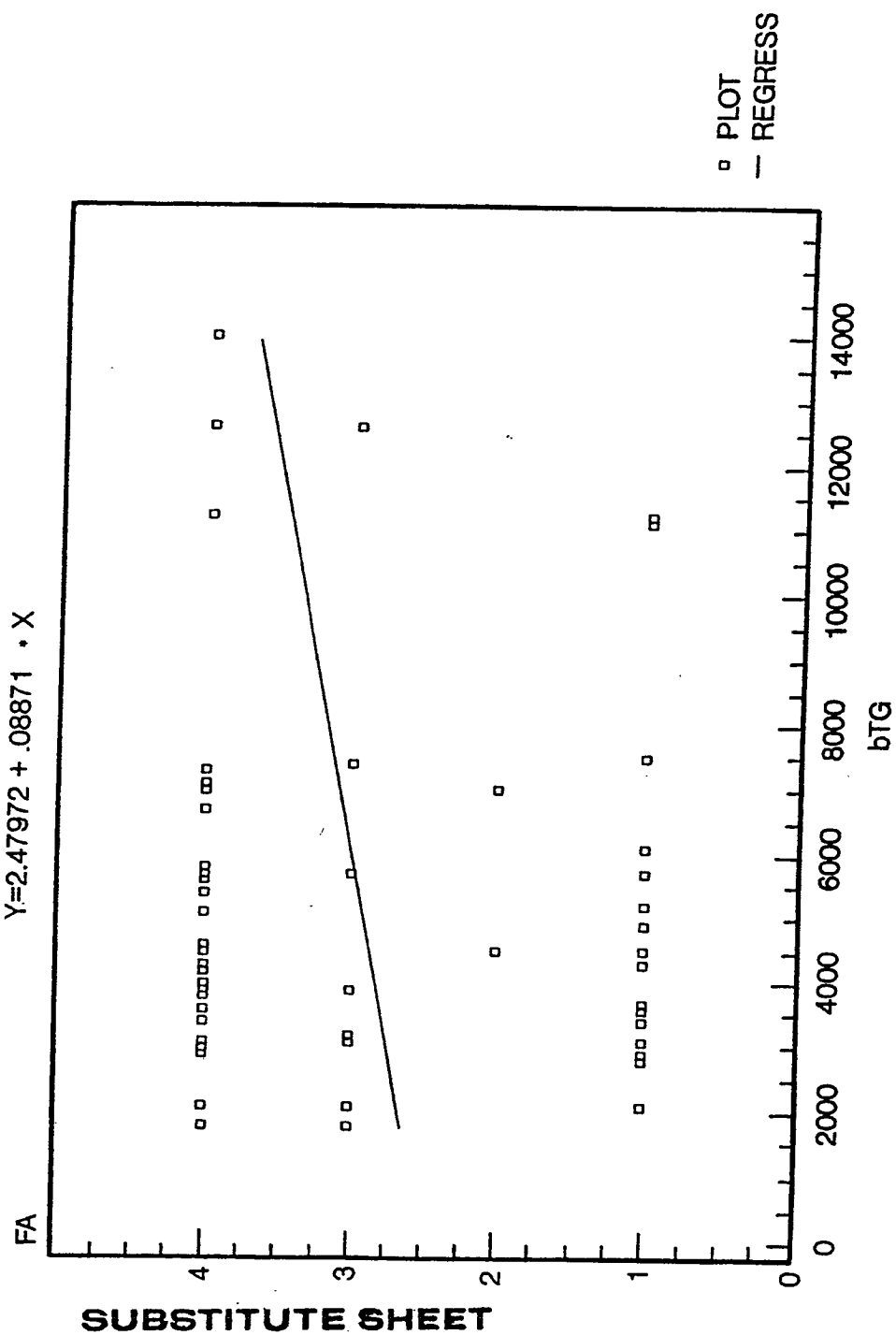
releasate sample to a predetermined range  
of amounts of the identical activity of  
platelet releasate products; and  
(ii) a pharmaceutically acceptable carrier  
or diluent for said platelet releasate.

5

41. The method of claim 40 wherein said composition  
is substantially free of blood or plasma contaminants  
and platelet ghosts or other material contained in  
platelets but not released by said platelets.

Fig. 1

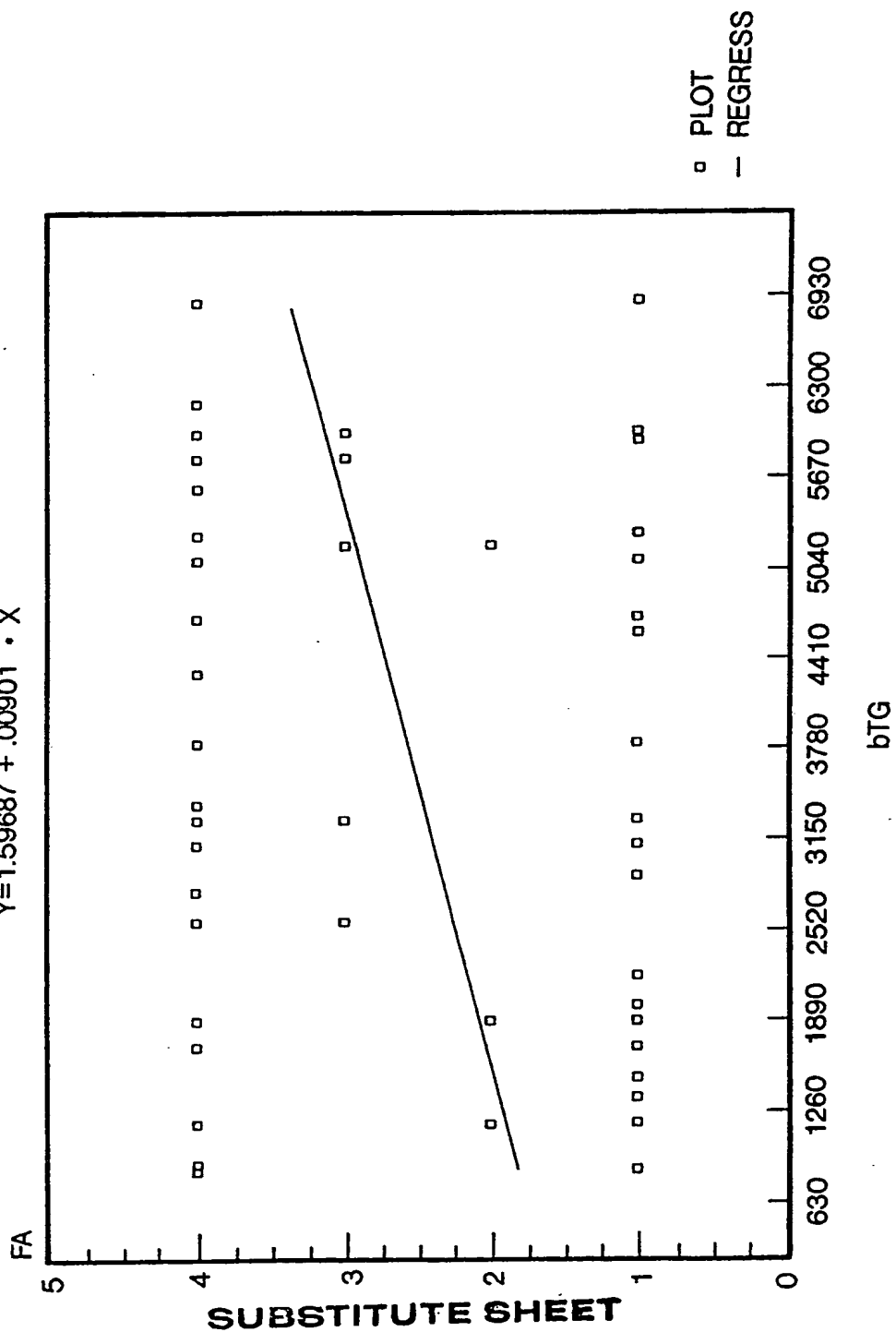
$$Y_i = 2.47972 + .08871 \cdot X$$



2/6

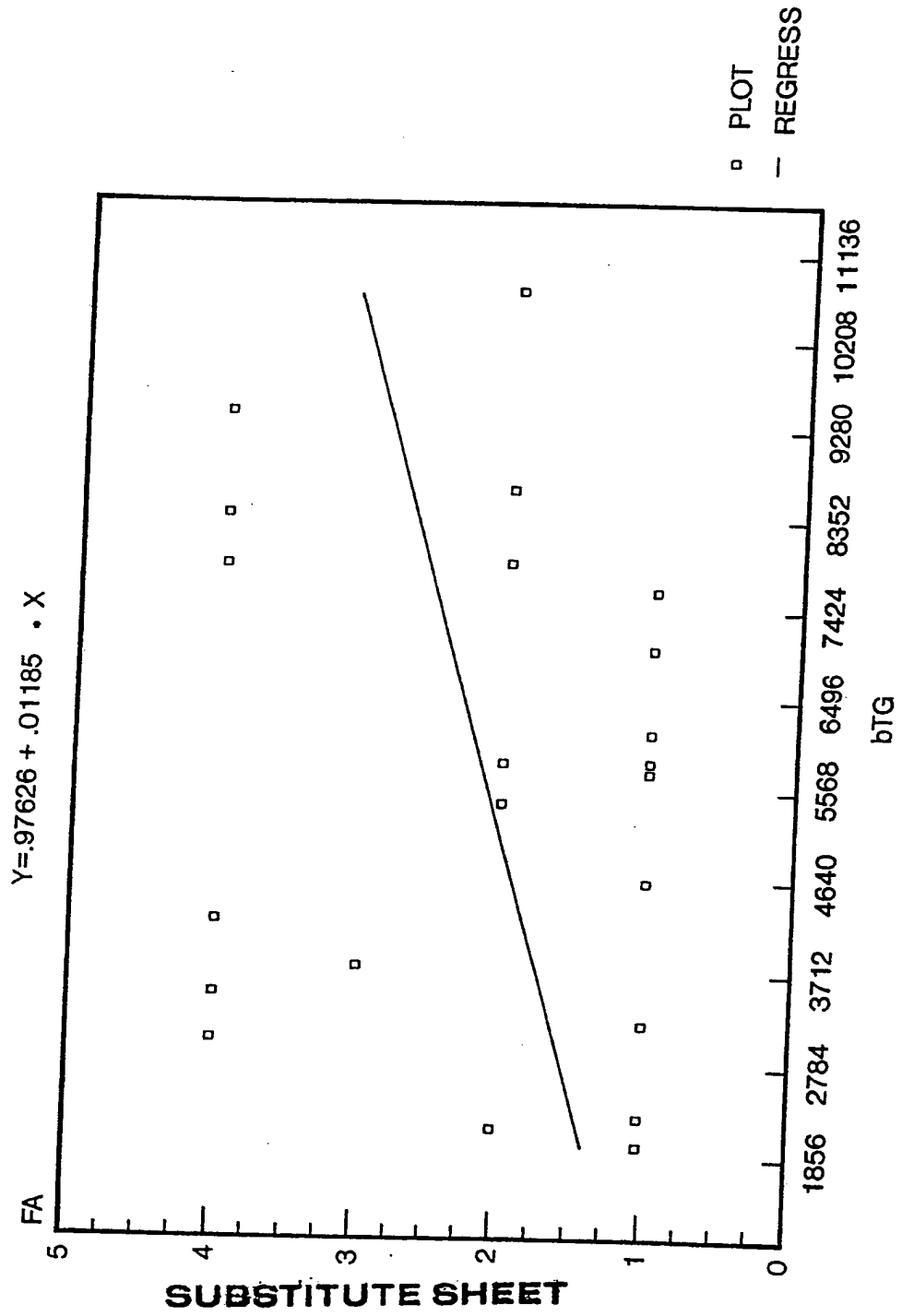
Fig. 2

$$Y = 1.59687 + .00901 \cdot X$$



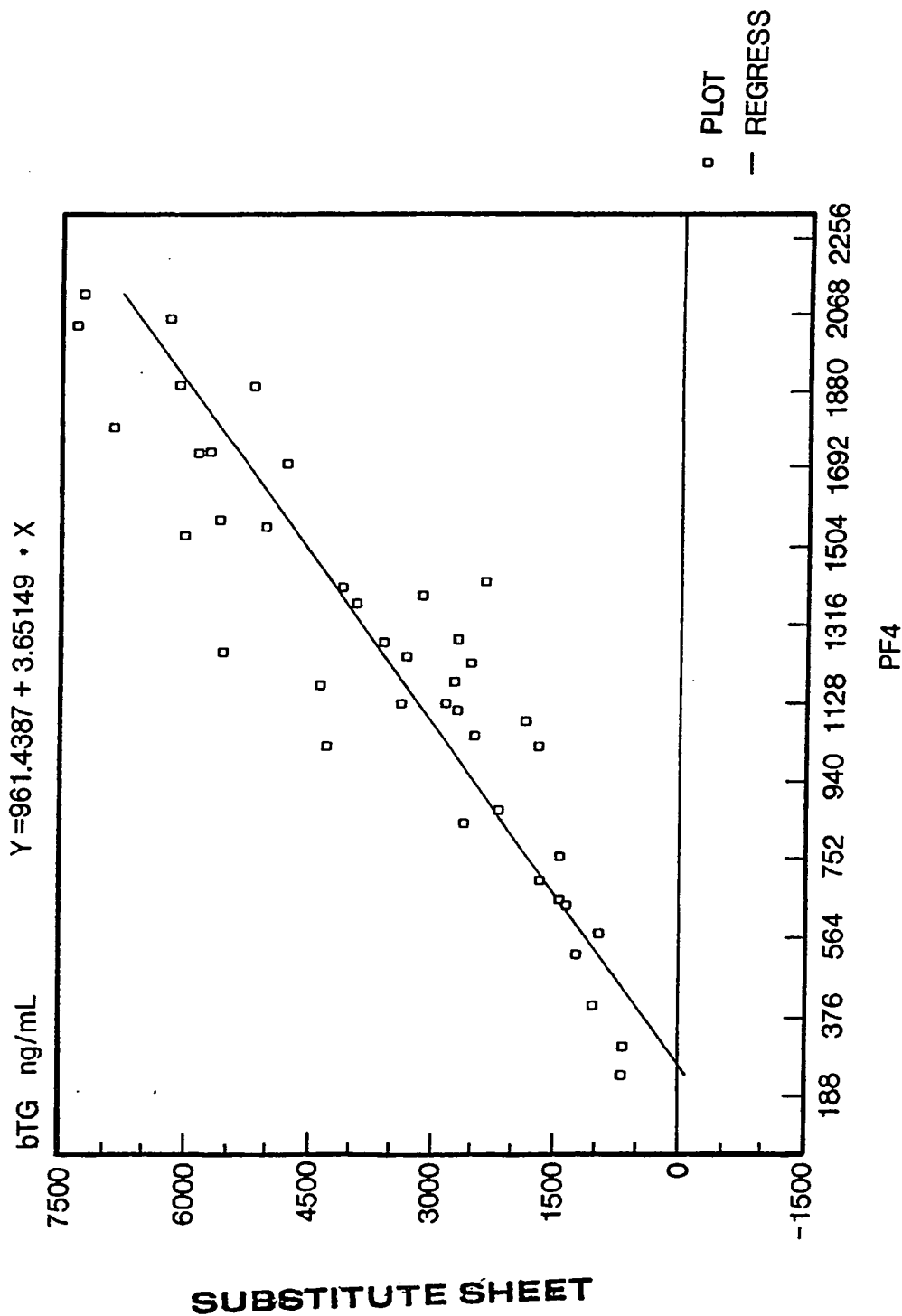
3/6

Fig. 3



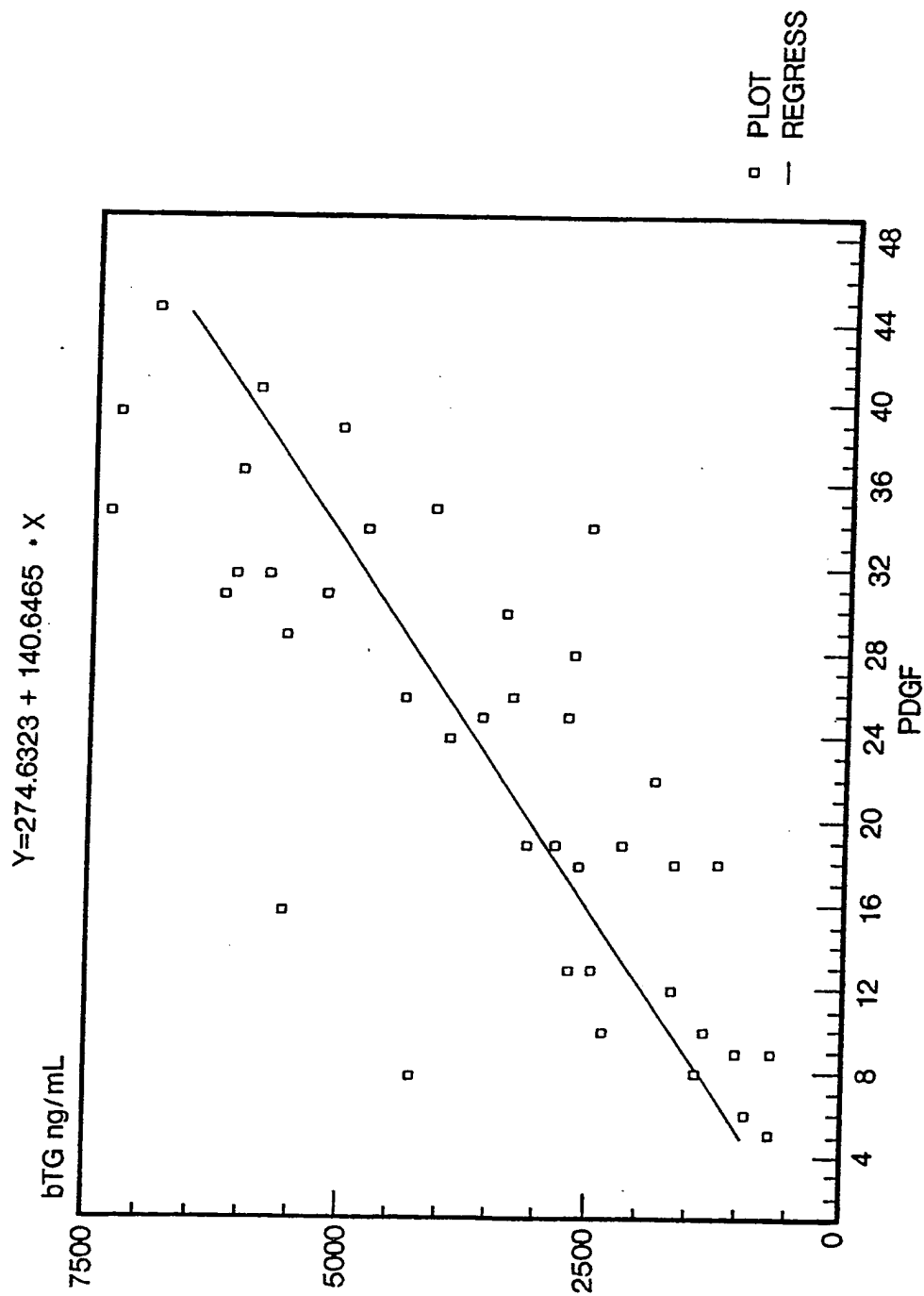
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Fig. 4



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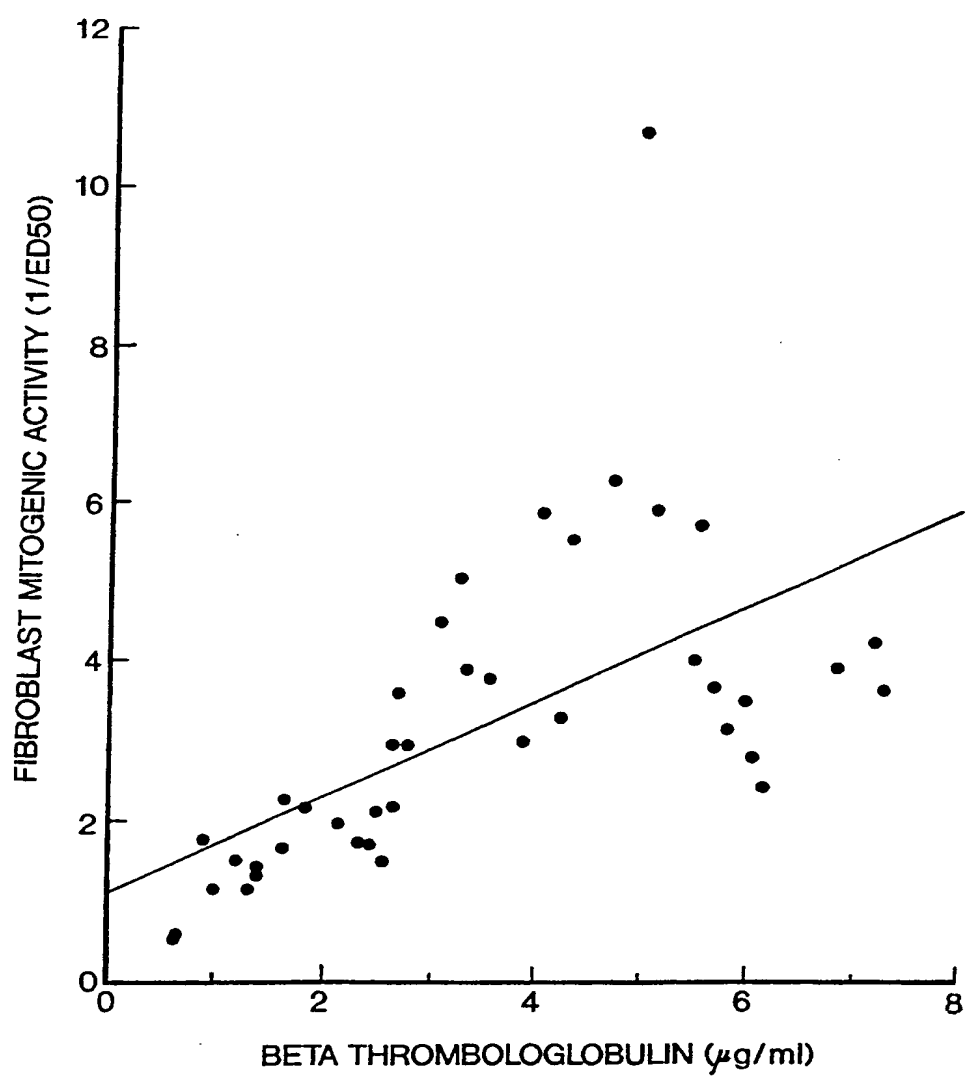
Fig. 5



SUBSTITUTE SHEET



6/6

*Fig. 6***SUBSTITUTE SHEET**

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/05301

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): A61K 35/14 U.S. Cl: 424/532														
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched <sup>4</sup></div> <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;">Classification System</td> <td style="width: 50%; border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none; padding-top: 10px;">U.S. Cl. 424/532</td> <td style="border: none;"></td> </tr> </table> <div style="text-align: center; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>5</sup></div> <p style="margin-top: 10px;">Chemical Abstracts: "Platelet releasate"</p>			Classification System	Classification Symbols	U.S. Cl. 424/532									
Classification System	Classification Symbols													
U.S. Cl. 424/532														
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category <sup>6</sup></th> <th style="width: 70%;">Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup></th> <th style="width: 20%;">Relevant to Claim No. <sup>18</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">X/Y</td> <td style="vertical-align: top;">US, A, 4,760,131, (SUNDSMO et al.) 26 July 1988, see col. 3, line 65 to col. 4, line 5.</td> <td style="text-align: center; vertical-align: top;">4,5/1-41</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">US, A, 4,195,072 (WORKMAN, Jr.) 25 March 1980, see col. 1, line 63 to col. 2, line 50.</td> <td style="text-align: center; vertical-align: top;">1-41</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">Intl. Journal Of Tissue Real., Volume X(6) issued 1988, G. R. Grotendorst, "Growth Factors as Regulators of Wound Repair", pages 337-344, see entire document.</td> <td style="text-align: center; vertical-align: top;">1-41</td> </tr> </tbody> </table>			Category <sup>6</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>	X/Y	US, A, 4,760,131, (SUNDSMO et al.) 26 July 1988, see col. 3, line 65 to col. 4, line 5.	4,5/1-41	Y	US, A, 4,195,072 (WORKMAN, Jr.) 25 March 1980, see col. 1, line 63 to col. 2, line 50.	1-41	Y	Intl. Journal Of Tissue Real., Volume X(6) issued 1988, G. R. Grotendorst, "Growth Factors as Regulators of Wound Repair", pages 337-344, see entire document.	1-41
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>15</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATION</b> <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; padding: 5px;">           Date of the Actual Completion of the International Search <sup>2</sup>   <div style="text-align: center; font-size: 1.2em;">26 October 1990</div> </td> <td style="width: 50%; border: none; padding: 5px;">           Date of Mailing of this International Search Report <sup>2</sup>   <div style="text-align: center; font-size: 1.2em;">28 DEC 1990</div> </td> </tr> <tr> <td style="width: 50%; border: none; padding: 5px;">           International Searching Authority <sup>1</sup>   <div style="text-align: center;">ISA/US</div> </td> <td style="width: 50%; border: none; padding: 5px;">           Signature of Authorized Officer <sup>20</sup>  <div style="text-align: center;">              Jane Williams           </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <sup>2</sup>  <div style="text-align: center; font-size: 1.2em;">26 October 1990</div>	Date of Mailing of this International Search Report <sup>2</sup>  <div style="text-align: center; font-size: 1.2em;">28 DEC 1990</div>	International Searching Authority <sup>1</sup>  <div style="text-align: center;">ISA/US</div>	Signature of Authorized Officer <sup>20</sup> <div style="text-align: center;">              Jane Williams           </div>								
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